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(71) Applicant: CHILDREN'S HOSPITAL MEDICAL CENTER [US/US]; 3333 Burnet Avenue, Cincinnati, OH 45229 (US).

(72) Inventors: WEAVER, Timothy, Edward; 1217 Fawn Court, Loveland, OH 45140 (US). AKINBI, Henry, Toyin; 346 Fleming Road, Cincinnati, OH 45215 (US).

(74) Agents: JOSEPHIC, David, J. et al.; Wood, Herron & Evans, L.L.P., 2700 Carew Tower, Cincinnati, OH 45202 (US).

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(54) Title: LYSOZYME FUSION PROTEINS IN INFECTIONS

(57) Abstract

A method and composition for prophylaxis and/or therapeutic treatment of bacterial infections, particularly respiratory bacterial infections. A fusion protein of lysozyme and the carboxyl terminal propeptide of surfactant protein–B (SP–B) with the preceding ten amino acids of the mature SP–B peptide, or recombinant lysozyme alone, is administered in a pharmaceutically acceptable medium to an individual. The fusion protein or recombinant lysozyme may be selected so as to deliver it to a target infection site, such as the lungs or gastrointestinal tract. The method and composition eliminates problems associated with conventional antibiotic treatments, such as inefficacy and promotion of antibiotic resistant bacterial strains.

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LYSOZYME FUSION PROTEINS IN INFECTIONS

Related Applications

This application is a Continuation-In-Part of U.S. Application Serial No. 09/193,877 filed November 18, 1998, now pending.

The U.S. Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided for by the terms of Grant Nos. R01-HL56285 and HL56285S awarded by the National Institutes of Health.

Field of the Invention

The invention relates to prophylactic and therapeutic uses of recombinant lysozyme and a lysozyme/surfactant protein-B fusion protein in bacterial infections.

Background of the Invention

Bacterial infections remain a leading cause of worldwide morbidity and mortality. While antibiotics administered to treat bacterial infecti ns are often saf and efficacious, there is widespread concern over bacterial strains

that have become resistant to classic antibiotic treatment regim ns. In individuals infected with resistant strains, antibiotic administration results in incomplete and ineffective treatment, necessitating additional treatment along with propagation of the resistant strains. Preventative measures and alternative treatments that do not rely on antibiotics are therefore desirable.

In certain individuals such as those who are immunocompromised, who are in less than optimal health, who lack fully functional immune systems such as neonates or geriatric patients, or who suffer from a disease affecting the respiratory tract such as cystic fibrosis or the gastrointestinal tract such as ulcerative colitis or sprue, bacterial infections may have severe consequences leading to serious illness or even death. For example, production of altered mucus in cystic fibrosis patients leads to dilation of the exocrine ducts, destruction of acinar tissue, and replacement of the destroyed tissue by fibrous connective tissue. Involvement of the lungs leads to pneumonia and bronchiectasis. The paucity of systemic dissemination of infection in these patients, even in the presence of substantial bacterial colonization of the lungs, indicates that their systemic immunity is essentially intact, yet they are susceptible to pulmonary infections. These patients often die in their teens or early twenties from terminal lung infections in spite of aggressive antibiotic therapy.

The respiratory and gastrointestinal tracts are frequent sites of bacterial infections in normal individuals. The normal respiratory tract has natural clearance mechanisms that help to prevent bacterial colonization. These mechanisms include the presence of a mucus gel that acts as a barrier

to bacterial invasion, the propulsive forces of the cilia on the epithelial lining of th airways, and the secretion of antibacterial humoral factors such as the secretory immunoglobulins IgA and IgM, the proteins lactoferrin, betalysin and fibronectin, complement components and the enzyme lysozyme. Of these antimicrobial factors, lysozyme is the best established antimicrobial substance in airway secretions.

Human lysozyme is a naturally occurring enzyme that is known to exhibit bactericidal activity *in vitro* and thus would appear to be a promising way to prevent and/or treat bacterial infections. Lysozyme is a small (15 kilodaltons), basic protein that is produced in most tissues. It is secreted and is present in most body secretions such as mucus. In the lungs, immunohistochemical methods have localized lysozyme to the bronchial serous submucosal glands, alveolar macrophages and lamellar bodies in Type II alveolar epithelial cells. Approximately 80% of lysozyme secreted into the airway comes from the mucosal layer of the upper airways.

In vitro, lysozyme has been demonstrated to act independently to cause bacterial death. It is known that one way lysozyme kills bacteria is by hydrolyzing the glycosidic bond between C-1 of N-acetylmuramic acid and C-4 of N-acetylglucosamine in the bacterial polysaccharide cell wall. Lysozyme can also kill bacteria by acting synergistically with other proteins such as complement or antibody to lyse bacterial cells. Lysozyme, produced by polymorphonuclear leukocytes such as neutrophils, inhibits chemotaxis of polymorphonuclear l ukocytes and limits th production of xygen free radicals following an infection. This limits the degree of inflammation, while at the

same time enhances phagocytosis by these cells. Lysozyme is also probably involved in the response of airway tissue to injury.

While the antibacterial effects of lysozyme *in vitro* have been well documented, there has heretofore been no way to exploit these effects of lysozyme for *in vivo* use. Previous reports furthermore implied that sustained lysozyme administration would be harmful.

Pulmonary surfactant, a complex mixture of phospholipids and proteins, is synthesized and secreted by alveolar type II epithelial cells, a specialized exocrine cell. Surfactant protein-B (SP-B) is one of the protein components of pulmonary surfactant. Normal respiratory function requires pulmonary surfactant for maintenance of alveolar patency.

A method and composition for the prophylaxis and/or therapeutic treatment of bacterial infections, particularly respiratory bacterial infections as frequently occurs in patients with cystic fibrosis, is thus desirable.

Summary of the Invention

This invention is directed to a composition for the prophylaxis or therapeutic treatment of a bacterial infection in a mammal. The composition is either a lysozyme/surfactant protein-B (SP-B) fusion protein SEQ ID NO:3 (rat lysozyme SEQ ID NO:1 fused with the carboxyl terminal SP-B propeptide and the preceding ten amino acids of the mature SP-B peptide SEQ ID NO:4), or a lysozyme/SP-B fusion protein SEQ ID NO:6 (human lysozyme SEQ ID NO:5 fused with the carboxyl terminal SP-B propeptide and the preceding ten amino acids of th mature SP-B peptide SEQ ID NO:4), or recombinant lysozym alone SEQ ID NO:1. The composition prevents or treats a respiratory

infection such as occurs frequently in individuals with cystic fibrosis, or may occur in individuals having other types of respiratory diseases, or a gastrointestinal infection. The invention's use of recombinant lysozyme reduces mortality and enhances *in vivo* clearance of bacteria such as *Pseudomonas aeruginosa*, which is the major airway pathogen in patients with cystic fibrosis. Furthermore, the elevated lysozyme activity in bronchoalveolar lavage fluid resulting from administration of lysozyme is not associated with altered lung function or structure, or with chronic inflammatory disease.

The invention is also directed to a method of preventing or treating a bacterial infection in a mammal by administering a lysozyme/SP-B fusion protein SEQ ID NO:3 or SEQ ID NO:6 in a pharmaceutically acceptable composition in a dosing regimen sufficient to prevent or treat the infection. The route of administration may be parenteral, for example by inhalation, or nonparenteral.

The invention is still further directed to a fusion protein SEQ ID NO:3, comprising a rat lysozyme SEQ ID NO:1 and a carboxyl terminal SP-B propeptide with the ten terminal amino acids of the mature peptide SEQ ID NO:4, having antibacterial activity in a mammal.

The invention is still further directed to a fusion protein SEQ ID NO:6, comprising a human lysozyme SEQ ID NO:5 and a carboxyl terminal SP-B propeptide with the ten terminal amino acids of the mature peptide SEQ ID NO:4, having antibacterial activity in a mammal.

Th invention is additionally directed to a method of treating a bacterial r spiratory infection in an individual having cystic fibrosis by

administering a lysozyme/SP-B fusion protein SEQ ID NO:3 or SEQ ID NO:6 to the individual. The fusion protein may be administered by aerosol installation and/or inhalation.

The invention is also directed to a method of preventing or treating a bacterial infection in a mammal by administering recombinant lysozyme SEQ ID NO:1 in a pharmaceutically acceptable composition at a dose sufficient to prevent or treat the infection, such as treating a bacterial infection in a patient with cystic fibrosis.

The invention is additionally directed to a composition comprising recombinant lysozyme SEQ ID NO:1 having antibacterial activity.

These and other methods and compositions will be apparent in light of the following figures and detailed description.

Brief Description of the Figures

FIG. 1 is a histogram showing bacterial clearance from lungs of transgenic (lysozyme/surfactant protein-B fusion protein) and control mice.

FIG. 2A is a photograph showing expression of recombinant lysozyme SEQ ID NO:1 in a transgenic mouse line probed with mouse lysozyme complementary DNA (cDNA), and FIG 2B is a photograph showing expression of recombinant lysozyme SEQ ID NO:1 in the same transgenic mouse line probed with rat cDNA.

FIG. 3 is a photograph showing analysis of lysozyme protein expression.

FIG. 4 is a hist gram of lysozyme activity in bronchoalveolar lavage (BAL) fluid.

FIG. 5A is a photograph showing lysozyme cellular localization in wild type mice, and FIG. 5B is a photograph showing lysozyme cellular localization in transgenic mice.

FIG. 6A is a photograph showing lung structure in transgenic mice that overexpress lysozyme, and FIG. 6B is a photograph showing lung structure in wild type mice.

FIG. 7 is a histogram illustrating the cellular composition of BAL fluid.

FIG 8 is a histogram showing clearance of Group B *Streptococcus* (GBS) from the lungs at six hours post-infection.

FIG 9 is a histogram showing clearance of *Pseudomonas aeruginosa* from the lungs at twenty-four hours post infection.

Detailed Description of the Preferred Embodiment

Preparation of Recombinant Lysozyme and SP-B and Fusion Protein

Rat lysozyme is a hydrophobic peptide of 148 amino acids SEQ ID NO:1. Human lysozyme SEQ ID NO:5 also has 148 amino acids and has 69% homology with rat lysozyme.

Surfactant protein-B (SP-B) is a hydrophobic peptide of 79 amino acids that avidly associates with surfactant phospholipids in the alveolar airspace. Human SP-B is synthesized by alveolar type II epithelial cells as a prepropeptide of 381 amino acids SEQ ID NO:2. Mature SP-B is generated by sequential cleavage of a 23 amino acid signal peptide, an amino terminal (N-terminal) propeptid of 177 amino acids, and a carb xyl terminal (C-terminal) propeptid of 102 amino acids. The C-terminal propeptid has been shown to

function in maintenance of the size of lamellar bodies which store SP-B and in determining th intracellular surfactant pool size.

Complementary DNA (cDNA) corresponding to rat lysozyme and human SP-B was synthesized. The protocol used for synthesis was that described in Akinbi et al., J. Biol. Chem. 1997; 272: 9640-9647, which is expressly incorporated by reference herein in its entirety. Complementary DNA to rat lysozyme was generated as follows. Alveolar Type II epithelial cells were isolated from adult rat lung as described by Dobbs, et al., An improved method for isolating Type II cells in high yield and purity, Am. Rev. Respir. Dis. 134:140-145, 1986). Total RNA was isolated from Type II cells by the method of Chirgwin, et al., The isolation of biologically active ribonucleic acid from sources enriched in ribonuclease, Biochemistry 18:5294-5299, 1979) and polyA$^+$ RNA by the method of Aviv and Leder, Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose, Proc. Natl. Acad. Sci. USA 69:1408-1412, 1972. Single stranded cDNA, generated from isolated polyA$^+$ RNA with reverse transcriptase (Maniatis, et al. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 1982), was used as a template for PCR amplification of the entire coding sequence of lysozyme using oligonucleotide primers based on the published sequence of the rat enzyme by Yeh, et al. Evolution of rodent lysozymes: Isolation and sequence of the rat lysozyme genes, Mol. Physiol. Evol. 2:25-75, 1993. Isolation of the human SP-B cDNA has previously been described (Glass r, S.W., et al. cDNA and

deduced amino acid sequence of human pulmonary surfactant-associated proteolipid SPL (Phe)., Proc. Natl. Acad. Sci. USA 84:4007-4011, 1987).

The synthesized cDNAs were either generated into a chimeric molecule consisting of the rat lysozyme protein and a carboxyl terminal (C-terminal) propeptide of a human surfactant protein B (SP-B) for evaluation, or recombinant rat lysozyme (SEQ ID NO:1) alone was generated. Specifically, this chimeric molecule was a fusion protein of residues 1-148 of rat lysozyme SEQ ID NO:1 and residues 270-381 of SEQ ID NO:2, shown in SEQ ID NO:4, forming a lysozyme/surfactant-B fusion protein SEQ ID NO:3.

Preparation of Transgenic Mice Overexpressing Lysozyme/SP-B Fusion Protein

Three lines of transgenic mice were generated that expressed a cDNA construct comprising the entire coding sequence for rat lysozyme SEQ ID NO:1 and the entire C-terminal propeptide of SP-B along with the preceding ten amino acids from the C-terminal of the mature peptide SEQ ID NO:4. The coding sequence for rat lysozyme SEQ ID NO:1 was cloned in frame with the coding sequence for the C-terminal propeptide and preceding 10 amino acids for human pulmonary surfactant protein B propeptide SEQ ID NO:4. FVB/N transgenic mice expressing a transgene construct encoding the fusion protein SEQ ID NO:3 under the control of the 3.7 kilobase (kb) human surfactant protein C (SP-C) promoter were generated as described in Lin et al. (J. Biol. Chem. 1996; 271: 19689-19695) which is expressly incorporated by reference herein in its entirety. The expression of transgene RNA in these mice was restricted to th distal respiratory epithelium.

Expression of the chimeric protein SEQ ID NO:3 was confirmed by Western blot analysis, as is known to one skilled in the art, using antibody #R96189 generated and characterized as reported by Lin et al., Structural requirements for intracellular transport of pulmonary surfactant protein B (SP-B) (Biochim. Biophys. Acta Mol. Cell. Res. 1312:177-185, 1996), that detects the C-terminal propeptide of SP-B proprotein. A protein of approximately 29 kDa was detected in transgenic mice using this antibody, as would be predicted by the size of the construct of 15 kDa rat lysozyme and 14 kDa C-terminal propeptide and preceding 10 amino acids SEQ ID NO:3. The transgene product was detected in both lung homogenates and in bronchoalveolar lavage (BAL) fluids, consistent with secretion of the chimeric protein SEQ ID NO:3 into the alveolar space. Constitutive expression of the chimeric protein SEQ ID NO:3 was not associated with altered lung structure, as assessed by light microscopy evaluation of lung tissue stained with hematoxylin and eosin.

Preparation of Transgenic Mice Overexpressing Lysozyme

In order to assess the role of lysozyme in pulmonary host defense, transgenic mouse lines were generated in which rat lysozyme SEQ ID NO:1 was targeted to the distal airway epithelium under the direction of the 3.7 kb human surfactant protein C (SP-C) promoter. Seven of twenty-one offspring from fertilized oocyte injections were positive for the transgene as assessed by PCR and confirmed by Southern blot analyses of tail DNA (now shown). Transgenic mice wer indistinct from wild type litt rmates with respect to body weight, lung weight, longevity and reproductive capability. Two

transgenic lines (3.5 and 2.6) had increased levels of lysozyme protein in br nchoalveolar lavage fluid.

Efficacy of Lysozyme/SP-B Fusion Protein

Five-week old transgenic mice (n=56) carrying the fusion protein SEQ ID NO:3 (treated) and their wild type litter mates (control) were infected with 10^6 strain III group B *Streptococci* (GBS) via intratracheal administration. Aliquots of GBS were grown in Todd Hewitt broth at 37°C overnight and bacteria were pelleted by centrifugation. The bacterial pellet was suspended in sterile phosphate buffered saline (PBS) at a concentration of 10^7/ml. One hundred microliters of bacterial suspension (10^6 bacteria) were drawn into tuberculin syringes fitted with 27 gauge needles in preparation for the intratracheal injection. All injections were carried out in a sterile environment.

Five to six week old mice maintained in clean rooms were used for bacterial clearance studies. Mice were anesthetized with a mixture of nitrous oxide and oxygen. The trachea was exposed through a midline incision and dissection through the thyroid gland. One hundred microliters of bacterial suspension was instilled into the trachea with a 27 gauge needle just below the cricoid cartilage. The two halves of the thyroid muscles were apposed at the midline and the skin incision was closed by approximating the two edges with surgical glue. Animals were housed for either 6 or 24 hours prior to sacrifice.

After either 6 or 24 hours post infection, lungs from treated and control mice were harvested, weighed, homogenized in PBS and plated on BAP to evaluate formation of GBS colonies. The cultured plates were incubated at

37°C for approximately 16-18 hours (overnight). Colony forming units per gram of lung tissue (CFU/g) were assessed by manual counting of bacterial colonies.

As shown in FIG. 1, transgenic mice harboring the chimeric protein SEQ ID NO:3 had significantly fewer CFU/g of lung tissue at both 6 hours and 24 hours following infection with GBS. The number of CFU in most transgenic mice was even less than the number of bacteria that had been administered. As shown in FIG. 1, transgenic mice had significantly enhanced clearance of GBS from the lungs at both the 6 h and 24 h post infection time points. At 6 h post-infection, BAP inoculated with lung tissue from transgenic (treated) mice had $1.99 \pm 1.4 \times 10^4$ CFU/g of tissue, while BAP inoculated with lung tissue from wild type (control) mice had $25.49 \pm 12.43 \times 10^4$ CFU/g ($p < 0.006$). At 24 h post-infection, BAP inoculated with lung tissue from transgenic (treated) mice had $9.9 \pm 6.43 \times 10^4$ CFU/g tissue, while BAP inoculated with lung tissue from wild type (control) mice had $67.29 \pm 34.2 \times 10^4$ CFU/g tissue ($p < 0.04$).

These results suggested that the expression of lysozyme/surfactant protein-B fusion protein SEQ ID NO:3 in the airway of the transgenic mice facilitated bacterial clearance from the airway. The results show that in mice carrying the lysozyme/surfactant protein-B fusion protein SEQ ID NO:3, bacterial proliferation was inhibited at 6 hours post infection, while bacterial clearance was enhanced at 24 hours post infection. Transgenic mice which xpressed a lysozyme/surfactant protein B fusion protein SEQ ID NO:3 in the distal airway were twelve-fold mor fficient in clearing bacteria

in the airway than their wild type littermates. This lysozym -produced efficacy is particularly striking, since wild type mice are inherently very efficient in clearing bacteria in the airway.

Two other lines of transgenic mice expressed the lysozyme/surfactant protein-B chimeric protein SEQ ID NO:3 although at lower levels. Bacterial clearance in these lines was correspondingly lower, although still significantly greater than that in wild type control mice. Lysozyme enzyme activity was increased 40% (relative to wild type littermates) in bronchoalveolar lavage fluid of the transgenic line expressing the highest levels of the lysozyme/SP-B fusion protein SEQ ID NO:3. Since bacterial clearance was enhanced twelve-fold in this line, the antibacterial effect may have been conferred by the SP-B C-terminal propeptide with the preceding amino acids from the mature peptide SEQ ID NO:4 alone, or the result of the combined action of SP-B and lysozyme SEQ ID NO:3 components; alternatively the effect may be due to increased lysozyme SEQ ID NO:1 activity.

Analysis of Lysozyme Transgene Expression

RNA With reference to FIGS. 2A and 2B, expression of the lysozyme transgene was assessed by Northern blot analysis of 2 μg of total cellular RNA isolated from lung tissue of five-week old transgenic mice (line 3.5). These are shown in FIG. 2A lanes 4 and 5, and in FIG. 2B lanes 3 and 4. Control wild type littermates are shown in FIG. 2A lanes 1-3 and in FIG. 2B lanes 1 and 2. Both transgenic and control samples were probed with mouse complementary DNA (cDNA) (FIG. 2A) and rat cDNA (FIG. 2B). In FIG. 2A, endogenous mouse lysozyme was the larger of the 2 transcripts. The mous

cDNA probe, because of cross hybridization between rat lysozyme and mouse cDNA, detected rat lysozyme.

A cDNA probe specific for rat lysozyme SEQ ID NO:1 detected a ~1 kb transcript, the predicted size of the lysozyme transgene (FIG. 2B). When the same RNA samples were probed with a mouse lysozyme cDNA (FIG. 2A), both the larger endogenous mouse lysozyme mRNA and rat lysozyme mRNA were detected. The mouse lysozyme mRNA was used as an internal control for normalization of samples.

Protein Because rat and mouse lysozyme have very similar molecular weights, it was not possible to resolve the proteins by SDS-PAGE. Total levels of lysozyme (rat and mouse) were estimated by Western blot analysis of equal amounts of protein from lung homogenates or BAL fluid from five-week old transgenic mice and wild type littermate controls. FIG. 3 shows a Western blot analysis of 1 μg of total lung protein from transgenic mouse line 3.5 (lanes 4-6) and control wild type littermates (lanes 1-3). Proteins were fractionated by SDS-PAGE under non-reducing conditions, blotted onto a nitrocellulose membrane and incubated with anti-human lysozyme antibody, which detected both mouse and rat lysozyme (molecular weight of about 15 kD).

As shown in FIG. 3, mice from transgenic line 3.5 (lanes 4-6) had a four-fold increase in the level of lysozyme protein in both lung homogenate and BAL fluid over wild type littermates (lanes 1-3). Mice from transgenic line 2.6 had a two-fold increase in lysozyme protein compar d to control wild type littermates (not shown).

Lysozyme Enzyme Activity With reference to FIG. 4, lysozym enzyme activity was assessed in BAL fluid from five-week old transgenic mice from line 3.5 (number of animals (n) = 4) and line 2.6 (n = 4), and control wild type littermates (n = 7) by a turbidimetric assay. Ten nanograms of BAL fluid protein was incubated with *Micrococcus lysodeikticus* suspended at an optical density (O.D.) of 1 at 450 nm. Following one hour of incubation at 37°C, the change in O.D. of the suspension was determined. Purified chicken egg white lysozyme was used to generate a standard curve.

As shown in FIG. 4, mice from transgenic line 3.5 had a 17.5-fold increase in lysozyme activity compared to wild type mice (550 units/ng BAL protein versus 31 units/ng BAL protein in wild type mice). The data are mean ± standard error of the mean (SEM), with $p = <0.0001$ for wild type versus transgenic line 3.5, $p = <0.0001$ for wild type versus 2.6, and $p = <0.0008$ for transgenic line 3.5 versus transgenic line 2.6. As predicted from Western blot analyses, mice from transgenic line 2.6 had increased lysozyme enzyme activity (205 units/ng BAL protein) relative to wild type controls ($p = 0.02$), but lower activity relative to transgenic line 3.5.

Spatial Expression of Lysozyme

With reference to FIGS. 5A and 5B, cellular localization of lysozyme protein in transgenic and control mice was obtained. Paraffin-embedded lung sections from five-week old transgenic mice from line 3.5 (FIG 5B) and wild type control littermates (FIG. 5A) were immunostained with hematoxylin/eosin using an anti-human lysozym antibody which detects both rat and mouse lysozyme and were photographed at a magnification of 80 x.

The overall architecture of lung sections of transgenic mice were indistinct from wild type littermates. There was no evidence of pulmonary edema or vascular congestion, and inflammatory cells were not detected in lung sections from uninfected transgenic mice.

Lysozyme was detected in Type II cells in wild type and transgenic mice, with more intense staining in Type II cells from transgenic mice. Transgenic mice, in addition, have expression in bronchiolar epithelial cells. In wild type mice, endogenous expression of epithelial lysozyme was restricted to Type II cells, whereas in transgenic mice, lysozyme expression was equally prominent in non-ciliated bronchiolar cells and Type II cells.

Effect of Lysozyme Overexpression on Lung Structure

With reference to FIGS. 6A and 6B, gross histologic features of paraffin-embedded, hematoxylin/eosin-stained sections of lungs from uninfected five-week old transgenic mice (FIG. 6A) and control wild type littermates (FIG. 6B) were compared at a magnification of 40 x. Total and differential cell counts (Cytospin® preparations stained with Diff-Quick®) in BAL fluids were performed.

As shown in FIG. 7, there were no significant differences in either the total cell count or in the distribution of cell types recovered from BAL fluid in transgenic mice compared to wild type control littermates, with $p = 0.98$ for total cell counts, $p = 0.74$ for macrophages, and $p = 0.95$ for lymphocytes. Data are mean ± SEM.

Effect of Lysozyme Overexpression on Bacterial Pathogen Clearance from the Airway

Clearance of Group B *Streptococcus* (GBS). To determine if increased lysozyme levels in the airway enhanced clearance of bacteria from the lungs, bacterial counts in quantitative cultures of lung homogenates from transgenic mice and wild type littermate controls were compared following an intratracheal injection of 10^6 colony forming units (CFU) of GBS. All mice survived until sacrifice at six hours post infection.

The results, shown in FIG. 8, are expressed as CFU/g lung tissue ± SEM, with $p=0.0172$ in wild type ($n=20$) versus transgenic line 3.5 ($n=19$), and $p=0.19$ in wild type versus transgenic line 2.6 ($n=10$). Mice from transgenic line 3.5 had a three-fold enhancement of GBS clearance (2.1 ± 0.1 x 10^6 CFU/g lung tissue versus 6.8 ± 0.5 x 10^6 CFU/g lung tissue in wild type mice, $p=0.0172$). The incidence of systemic dissemination of infection, as assessed by growth of GBS on plates inoculated with splenic homogenates, was less in transgenic mice (27% versus 60%, $p=0.04$). Clearance of GBS from the lungs was enhanced less than two-fold in mice from transgenic line 2.6 (4.2 ± 0.8 x 10^6 CFU/g lung tissue versus 7.1 ± 0.6 x 10^6 in wild type mice, $p=0.19$).

Clearance of *Pseudomonas aeruginosa*. Transgenic and wild type mice received intratracheal injections with 10^7 CFU of *Pseudomonas aeruginosa*. All transgenic mice survived until sacrifice at 24 hours post-infection. In contrast, 20% of infected wild type mice died.

FIG. 9 shows CFU/g lung tissue ± SEM. Bacterial clearance was enhanced approximately thirty-fold in mice from transgenic line 3.5 ($n=10$)

($1.06 \pm 0.05 \times 10^6$ CFU/g lung tissue, compared to $3.24 \pm 0.41 \times 10^7$ in wild type littermate controls ($n=10$, $p=0.03$). Bacterial clearance was enhanced approximately six-fold in mice from transgenic line 2.6 ($n=10$) ($6.52 \pm 0.71 \times 10^6$ CFU/g lung tissue, $p=0.05$). Systemic bacterial dissemination was not detected in surviving mice at 24 hours post-infection.

The lysozyme/SP-B fusion protein SEQ ID NO:3 or SEQ ID NO:6 or recombinant lysozyme SEQ ID NO:1 of the invention may be used to treat and/or reduce bacterial colonization of the airway. The latter use would be extremely beneficial in treating individuals with cystic fibrosis, since chronic bacterial colonization of the major airways, particularly by *Pseudomonas aeruginosa*, with consequent debilitating exacerbations is the major cause of the morbidity and mortality suffered by cystic fibrosis patients.

Cystic fibrosis is a systemic disease in which mucus secretion is altered so that a viscid mucus is produced. Production of altered mucus leads to dilation of the exocrine ducts, destruction of acinar tissue, and replacement of the destroyed tissue by fibrous connective tissue. Involvement of the lungs leads to pneumonia and bronchiectasis. These patients often succumb at a young age to terminal lung infections with *Pseudomonas aeruginosa* in spite of aggressive antibiotic therapy. Therefore, the bactericidal activity of lysozyme or the lysozyme/surfactant protein-B fusion protein *in vivo* offers a potential therapeutic strategy for suppressing bacterial colonization of the airways in cystic fibrosis patients without compromising whatever degree of respiratory function the pati nt exhibits.

It will be appreciated that prophylaxis or therapeutic treatment by the method and composition of the invention may range from total prevention, reduction of the bacterial load, amelioration of the severity of, or elimination of a bacterial infection. The lysozyme/SP-B fusion protein SEQ ID NO:3 or SEQ ID NO:6 or recombinant lysozyme SEQ ID NO:1 may be used to protect against all bacterial strains which colonize the respiratory tract such as, for example, *Staphylococcus aureus*, *Streptococcus* species, *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Klebsiellae* species, *Proteus* species, *Pseudomonas cepacia*, *Haemophilus influenzae*, *Bordetella pertussis*, *Mycoplasma pneumoniae*, *Legionella pneumophila*.

Additionally, the invention may be used to combat gastrointestinal infections. The lysozyme/surfactant protein-B fusion protein SEQ ID NO:3 or SEQ ID NO:6 may be administered by an enteral route to target the gastrointestinal tract for treating or preventing infections with bacterial strains that colonize the gastrointestinal tract such as, for example, *Salmonellae* species, *Shigellae* species, *Escherichia coli*, and *Vibrio* species, *Yersinia enterocolitica*, *Campylobacter fetus*, ssp. *jejuni*, and *Helicobacter pylori*. The lysozyme/SP-B may be formulated for oral administered as a solid or liquid in the form of a capsule, tablet, syrup, and so on.

Other variations or embodiments of the invention will also be apparent to one of ordinary skill in the art from the above description. For example, other fusion proteins besides surfactant protein-B, such as members of the structurally related saposin protein family such as saposin A, saposin B, saposin C, saposin D, NK lysin, pore forming peptide of amoebapore A etc.,

could be generated. Various modes of administration besides inhalation could be used, such as injection, etc. The fusion protein SEQ ID NO:3 or SEQ ID NO:6 or recombinant lysozyme SEQ ID NO:1 may be administered either alone or in combination with antibiotic therapy. Thus, the forgoing embodiments are not to be construed as limiting the scope of this invention.

What is claimed is:

1. A composition comprising a lysozyme/surfactant protein-B fusion protein selected from the group consisting of SEQ ID NO:3 and SEQ ID NO:6.

2. A composition comprising recombinant lysozyme SEQ ID NO:1.

3. The composition of claims 1 or 2 for prophylaxis or therapeutic treatment of a bacterial infection in a mammal.

4. The composition of claims 1 or 2 for prophylaxis or therapeutic treatment of a respiratory bacterial infection.

5. The composition of claim 4 wherein said respiratory infection is in said mammal having cystic fibrosis.

6. The composition of claim 1 for prophylaxis or therapeutic treatment of a gastrointestinal infection in a mammal.

7. A fusion protein selected from the group consisting of SEQ ID NO:3 and SEQ ID NO:6 having antibacterial activity in a mammal.

8. A recombinant lysozyme SEQ ID NO:1 having antibacterial activity in a mammal.

9. A method of prophylaxis or therapeutic treatment of a bacterial infection in a mammal comprising administering a composition selected from the group consisting of a recombinant lysozyme SEQ ID NO:1 in a pharmaceutically acceptable carrier and a lysozyme/surfactant protein-B fusion protein selected from the group consisting of SEQ ID NO:3 and SEQ ID NO:6 in a pharmaceutically acceptable carrier to said mammal at a dose sufficient to prevent or treat the infection.

10. The method of claim 9 wherein the composition is administered by a method selected from the group consisting of inhalation and aerosol installation.

11. The method of claim 9 wherein the mammal is infected with *Pseudomonas*.

12. The method of claim 9 wherein the mammal has cystic fibrosis.

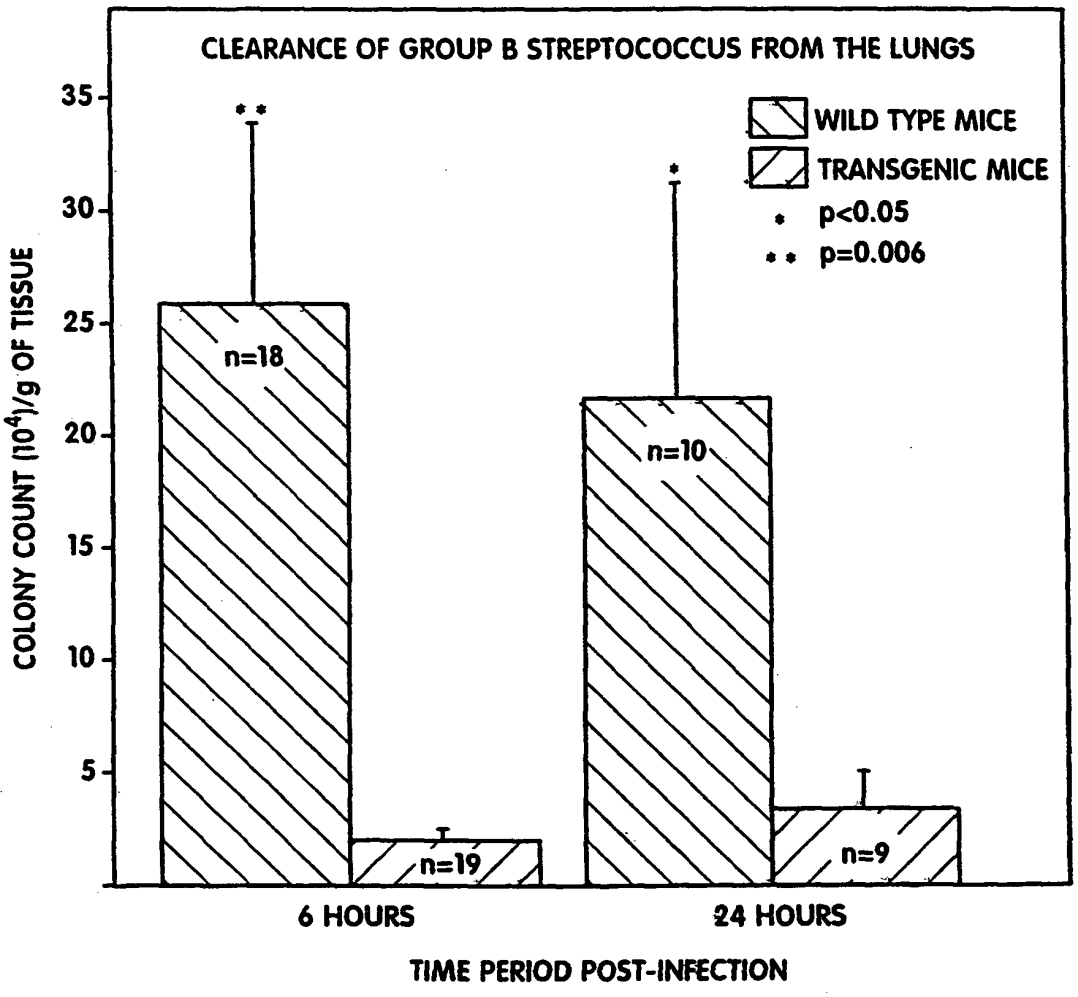


FIG. 1

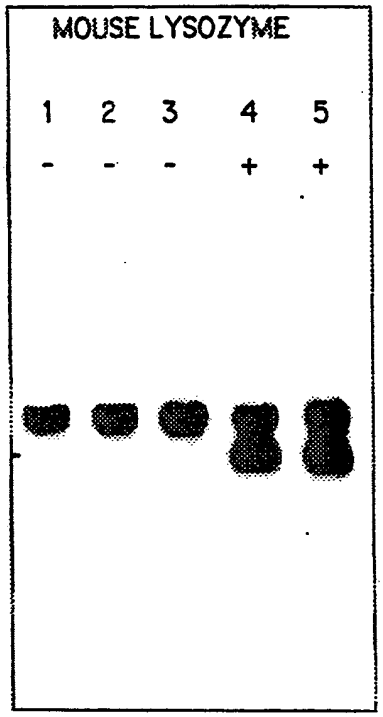


FIG. 2A

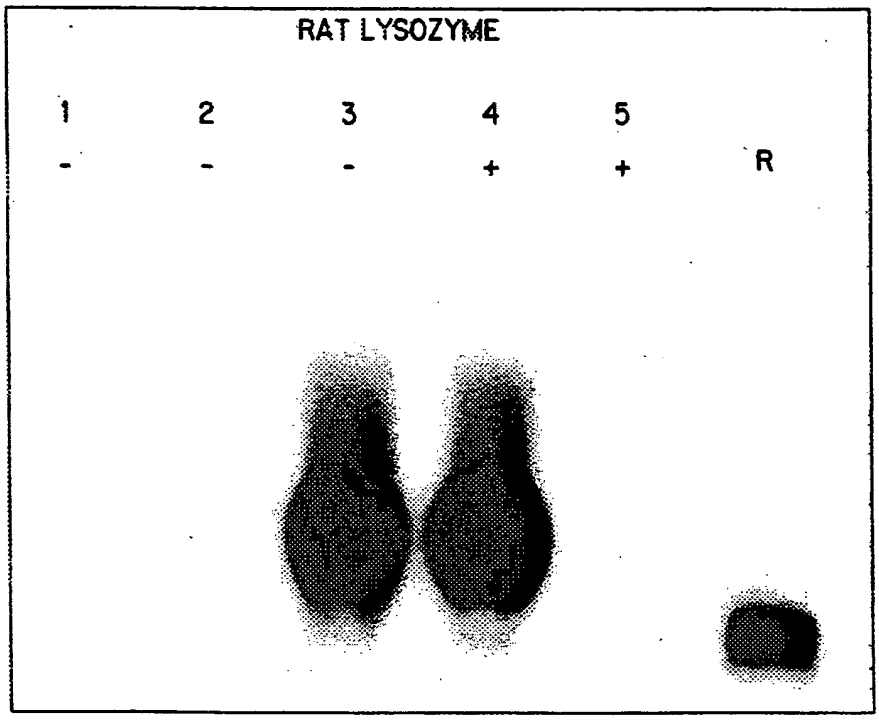


FIG. 2B

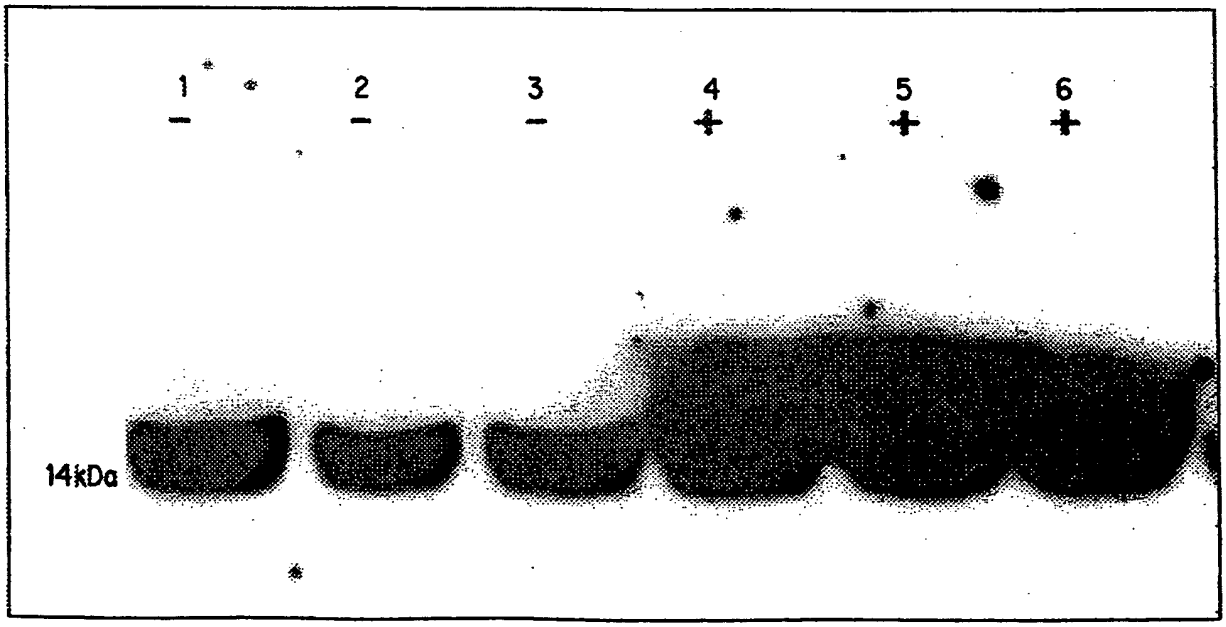
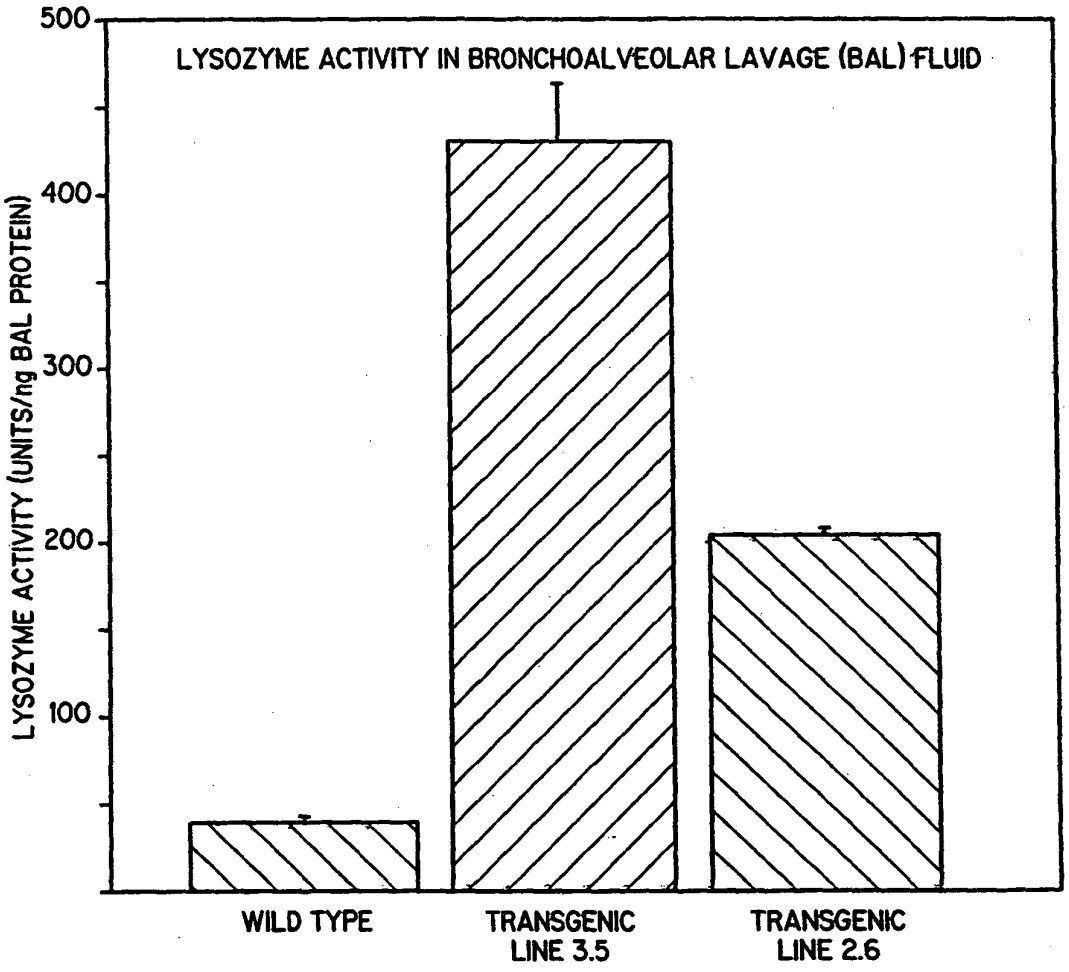


FIG. 3


LYSOZYME ACTIVITY IN BRONCHOALVEOLAR LAVAGE (BAL) FLUID
LYSOZYME ACTIVITY (UNITS/ng BAL PROTEIN)
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300
200
100
WILD TYPE
TRANSGENIC LINE 3.5
TRANSGENIC LINE 2.6

FIG. 4

FIG. 5B

FIG. 5A

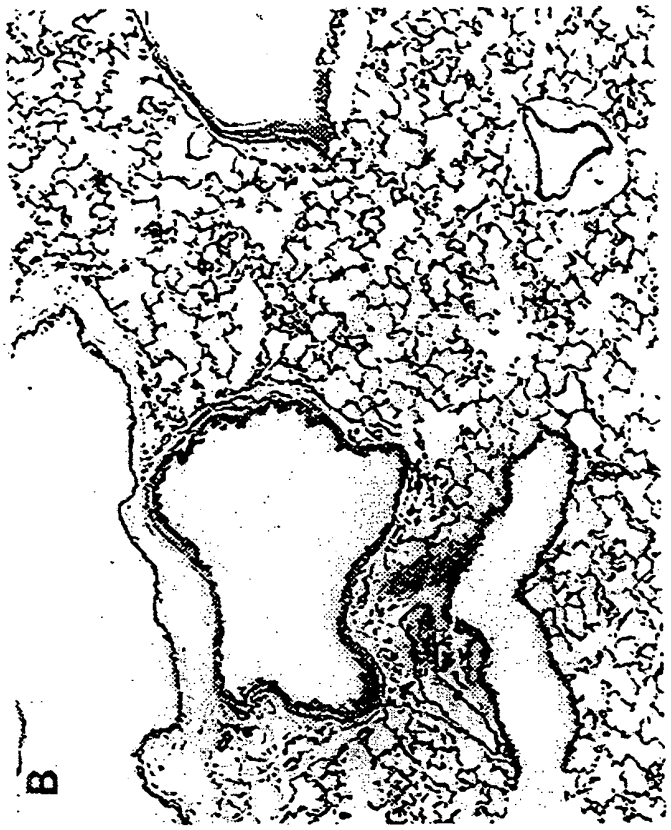


FIG. 6B

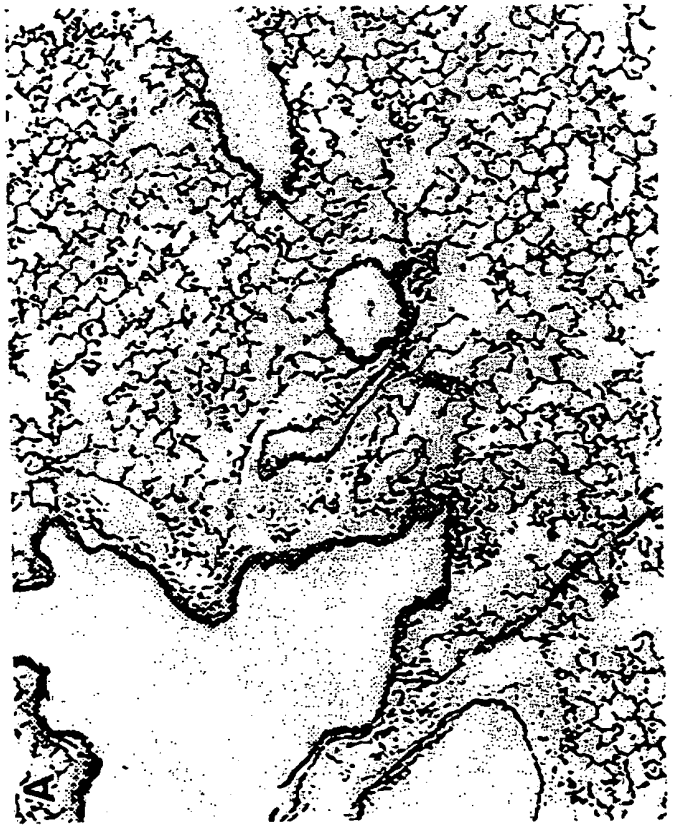
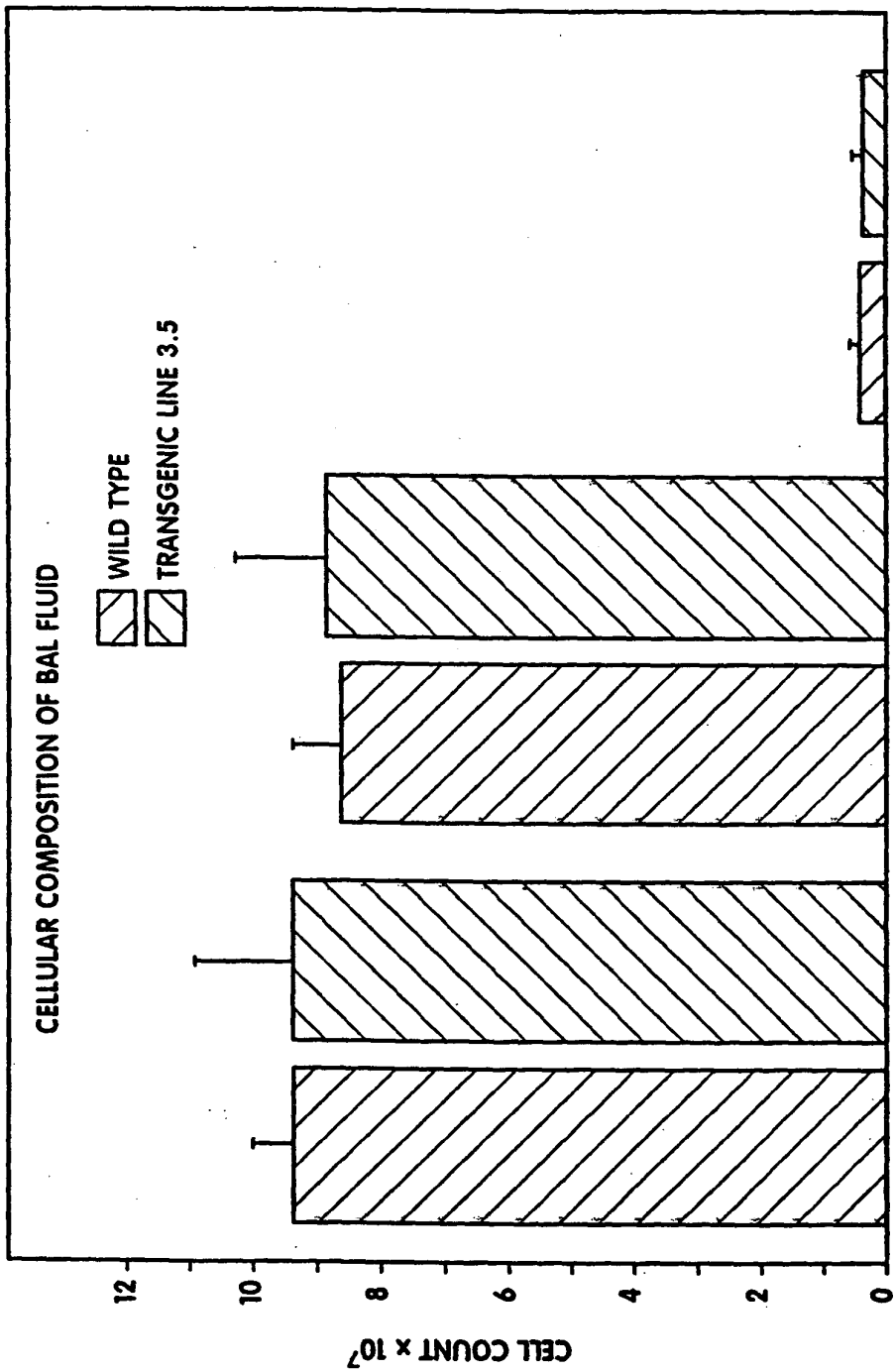


FIG. 6A


CELLULAR COMPOSITION OF BAL FLUID
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4
6
8
10
12
TOTAL COUNT
MACROPHAGES
LYMPHOCYTES

FIG. 7

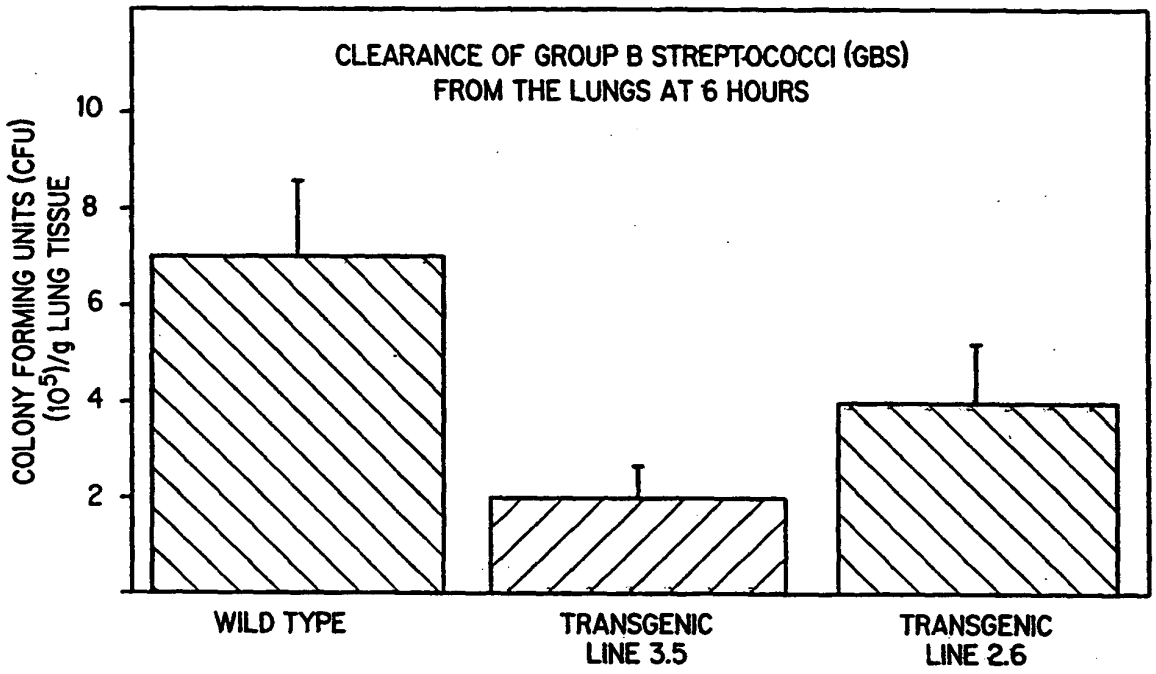

FIG. 8

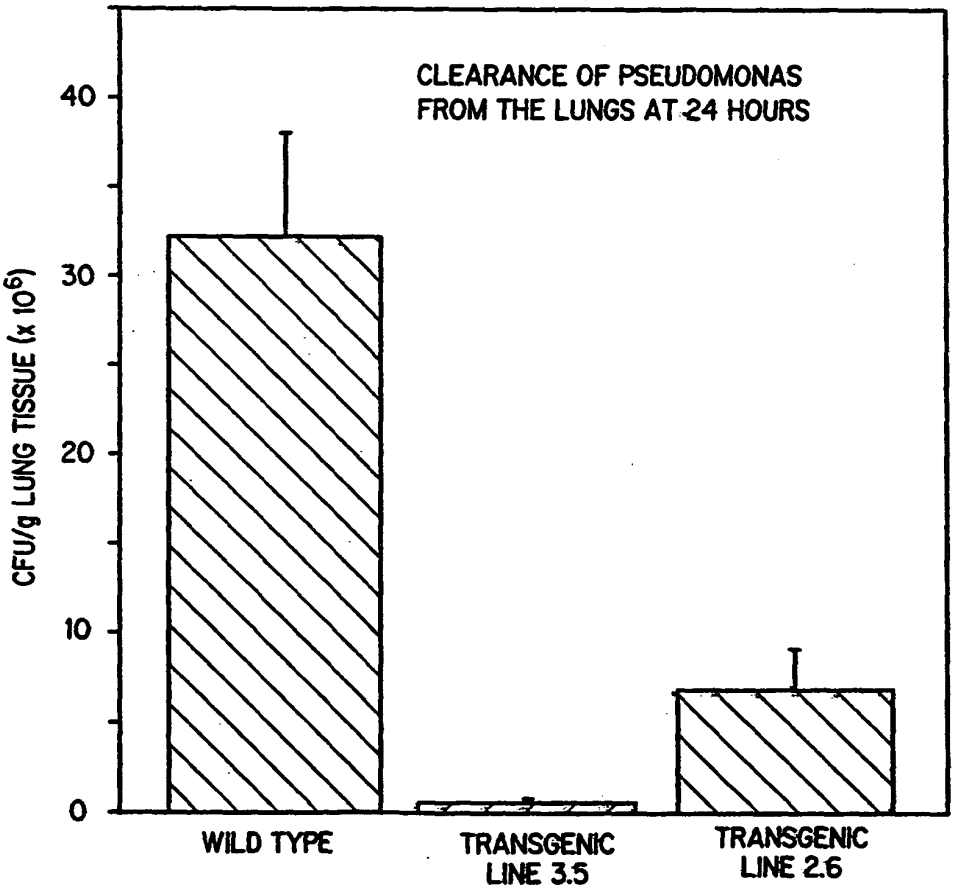

FIG. 9

SEQUENCE LISTING

<110> Weaver, Timothy E.

<120> LYSOZYME FUSION PROTEINS IN INFECTIONS

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Pro Gly Asp Gln Ser Thr Asp Tyr Gly Ile Phe Gln Ile Asn Ser Arg
65                  70                  75                  80
Tyr Trp Cys Asn Asp Gly Lys Thr Pro Arg Ala Lys Asn Ala Cys Gly
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Pro Lys Pro Leu Arg Asp Pro Leu Pro Asp Pro Leu Leu Asp Lys Leu
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Val Leu Pro Val Leu Pro Gly Ala Leu Gln Ala Arg Pro Gly Pro His
            180                 185                 190
Thr Gln Asp Leu Ser Glu Gln Gln Phe Pro Ile Pro Leu Pro Tyr Cys
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Trp Leu Cys Arg Ala Leu Ile Lys Arg Ile Gln Ala Met Ile Pro Lys
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225                 230                 235                 240
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            260                 265                 270
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Thr Gly Glu Trp Leu Pro Arg Asp Ser Glu Cys His Leu Cys Met Ser
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Leu Ala Gln His Glu Ser Asn Tyr Asn Thr Gln Ala Arg Asn Tyr Asn
    50                  55                  60
Pro Gly Asp Gln Ser Thr Asp Tyr Gly Ile Phe Gln Ile Asn Ser Arg
65                  70                  75                  80
Tyr Trp Cys Asn Asp Gly Lys Thr Pro Arg Ala Lys Asn Ala Cys Gly
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Ile Pro Cys Ser Ala Leu Leu Gln Asp Asp Ile Thr Gln Ala Ile Gln
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Cys Ala Lys Arg Val Val Arg Asp Pro Gln Gly Ile Arg Ala Trp Val
        115                 120                 125
Ala Trp Gln Arg His Cys Lys Asn Arg Asp Leu Ser Gly Tyr Ile Arg
    130                 135                 140
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        195                 200                 205
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        35                  40                  45
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    50                  55                  60
Lys Cys Lys Gln Phe Val Glu Gln His Thr Pro Gln Leu Leu Thr Leu
65                  70                  75                  80
Val Pro Arg Gly Trp Asp Ala His Thr Thr Cys Gln Ala Leu Gly Val
                85                  90                  95
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            100                 105                 110
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        35                  40                  45
Leu Ala Lys Trp Glu Ser Gly Tyr Asn Thr Arg Ala Thr Asn Tyr Asn
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Ala Gly Asp Arg Ser Thr Asp Tyr Gly Ile Phe Gln Ile Asn Ser Arg
65                  70                  75                  80
Tyr Trp Cys Asn Asp Gly Lys Thr Pro Gly Ala Val Asn Ala Cys His
                85                  90                  95
Leu Ser Cys Ser Ala Leu Leu Gln Asp Asn Ile Ala Asp Ala Ala Ala
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Cys Ala Lys Arg Val Val Arg Asp Pro Gln Gly Val Arg Ala Trp Ala
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        35                  40                  45
Leu Ala Lys Trp Glu Ser Gly Tyr Asn Thr Arg Ala Thr Asn Tyr Asn
    50                  55                  60
Ala Gly Asp Arg Ser Thr Asp Tyr Gly Ile Phe Gln Ile Asn Ser Arg
65                  70                  75                  80
Tyr Trp Cys Asn Asp Gly Lys Thr Pro Gly Ala Val Asn Ala Cys His
                85                  90                  95
Leu Ser Cys Ser Ala Leu Leu Gln Asp Asn Ile Ala Asp Ala Ala Ala
            100                 105                 110
Cys Ala Lys Arg Val Val Arg Asp Pro Gln Gly Val Arg Ala Trp Ala
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        195                 200                 205
Leu Asp Arg Glu Lys Cys Lys Gln Phe Val Glu Gln His Thr Pro Gln
    210                 215                 220
Leu Leu Thr Leu Val Pro Arg Gly Trp Asp Ala His Thr Thr Cys Gln
225                 230                 235                 240
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Ser Pro Asp Leu
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INTERNATIONAL SEARCH REPORT

Interr. nal Application No
PCT/US 99/27403

A. CLASSIFICATION F SUBJECT MATTER
IPC 7 C12N15/56 C12N15/12 C12N15/62 C12N9/36 C07K14/47
A61K38/47 A61P11/00 A61P31/04 //A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A61K A01K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 343 406 A (MEDICHEMIE AG) 29 November 1989 (1989-11-29) the whole document	2-4,8-11
X	EP 0 222 366 A (BOEHRINGER INGELHEIM INT) 20 May 1987 (1987-05-20) page A page 3, line 1 - line 13 page 4, line 23 - line 27 claims 20,21	2-4,8,9, 11
X	WHITE T.J. ET AL.: "Primary structure of rat lysozyme." BIOCHEMISTRY, vol. 16, 1977, pages 1430-1436, XP002134002 the whole document	2,3,8,9, 11

-/—

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 March 2000	10/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Galli, I

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CLANCY R. ET AL.: "Acute on chronic bronchitis: a model of mucosal immunology" IMMUN. CELL BIOL., vol. 73, 1995, pages 414-417, XP000891413 the whole document	1-12
A	GRIESE M. ET AL.: "Nebulization of a bovine surfactant in cystic fibrosis" EUR. RESP. J., vol. 10, 1997, pages 1989-1894, XP000891398 the whole document	1-12
A	AKINBI H.T. ET AL.: "Rescue of SP-B knockout mice with a truncated SP-B protein." J. BIOL. CHEM., vol. 272, no. 5, 11 April 1997 (1997-04-11), pages 9640-9647, XP002133931 the whole document	1-12
A	WO 90 07469 A (BENSON BRADLEY J ;WRIGHT JORAE (US)) 12 July 1990 (1990-07-12) abstract page 12	1-12

6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/ 27403

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 9-12 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

Inter... al Application No
PCT/US 99/27403

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0343406 A	29-11-1989	DE 3818094 C	20-07-1989
		JP 2019323 A	23-01-1990
EP 0222366 A	20-05-1987	DE 3540075 A	14-05-1987
		AT 71658 T	15-02-1992
		AU 601164 B	06-09-1990
		AU 6502986 A	14-05-1987
		DE 3683447 A	27-02-1992
		DK 538286 A	13-05-1987
		FI 864552 A,B,	13-05-1987
		GR 3003691 T	16-03-1993
		HU 43111 A,B	28-09-1987
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		PT 83719 A,B	01-12-1986
		US 5618712 A	08-04-1997
		ZA 8608540 A	27-07-1988
WO 9007469 A	12-07-1990	US 5006343 A	09-04-1991
		AT 109971 T	15-09-1994
		AU 638903 B	08-07-1993
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		CA 2006956 A	29-06-1990
		DE 68917603 D	22-09-1994
		DE 68917603 T	16-03-1995
		EP 0451215 A	16-10-1991
		HK 1006806 A	19-03-1999
		HU 211244 B	28-11-1995
		JP 2954343 B	27-09-1999
		JP 4503953 T	16-07-1992